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The Bionetics Corp. in Support of the
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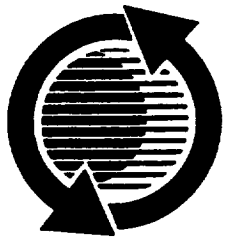

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Evaluation of Enzymatic Hydrolysis of CELSS Wheat Residue Cellulose at a Scale Equivalent to NASA's KSC Breadboard Project

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ABSTRACT

Biomass processing at the Kennedy Space Center CELSS breadboard project has focused on the evaluation of breadboard-scale enzymatic hydrolysis of wheat residue cellulose (25%, w/w). Five replicate runs of cellulase production by *Trichoderma reesei* (QM9414) and enzymatic hydrolysis of residue cellulose were completed. Enzymes were produced in 10 days (5 L, 25 g [dry weight] residue). Cellulose hydrolysis (12 L, 50 g [dry weight] residue) using these enzymes produced 5.5 to 6.0 g glucose liter^{-1} in 7 days. Cellulose conversion efficiency was 29%. These processes are feasible technically on a breadboard scale, but would only increase the edible wheat yield 10%.

THE PRIMARY GOAL of the KSC-CELSS Project is to integrate and evaluate various modules, subsystems, and components at a scale sufficient to demostrate feasibility for development and operation of a CELSS (KSC-CELSS Project Plan, W. M. Knott, unpublished document). To be successful, the project must demonstrate, among other objectives, that bioregenerative systems are feasible at an applicable scale, i.e., a one person scale for the Breadboard Project. The Breadboard Project encompasses operational concept and design validation, integration of components and subsystems into functional modules, and functional characterization of module and system operatons in a CELSS. The breadboard consists of three modules: biomass production, biomass processing and resource recovery, and food preparation. The research reported in this paper is a part of the biomass processing and resource recovery module.

FIGURE 1 diagrams the integration of inedible biomass processing into the KSC-CELSS breadboard during the time of the research reported in this paper. One of the stated project goals is to convert inedible to edible biomass. To accomplish a part of this goal, the biomass processing module includes blocks (bioreactors) for the production of cellulase enzymes and for the use of these enzymes to saccharify crop residue cellulose into glucose. The inclusion of these bioreactors was influenced by the seminal overiew by Petersen and Baresi (1) and from recommendations made in a 1986 internal report by the American Institute for Biological Sciences (AIBS)-KSC Biomass Processing Technical Panel entitled "Conversion of inedible wheat biomass to edible products for space missions".

The focus on cellulose conversion is further justified by the large quantity of this material in CELSS crop residues. TABLE 1 lists the composition of wheat grown in the KSC-CELSS breadboard Biomass Production Chamber (BPC). Cellulose comprises a small fraction, 15%, of the whole crop, but it is 25% of inedible residues, and 38% of inedible residues after extraction of water soluble components (leaching).

If a sizeable fraction of the insoluble cellulose, say 2/3 (of the 15% cellulose), could be converted to useful sugar, then the apparent harvest index could be increased from 40% to 50% (assumption: all sugar

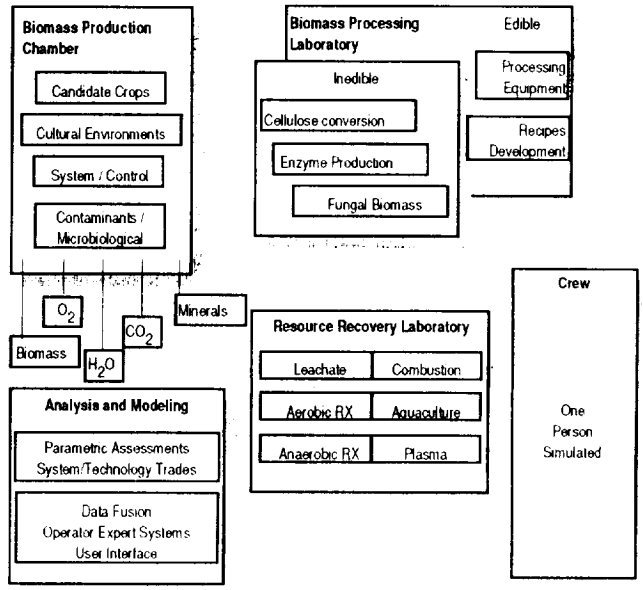

FIGURE 1. CONCEPTUAL DIAGRAM OF THE KSC CELSS BREADBOARD PROJECT.

TABLE 1. TYPICAL COMPOSITION OF BREADBOARD WHEAT AT HARVEST.

Component	Per Cent Composition	
Edible	40	
Inedible	60	
Water extractable (room temp, 2 hr)		
Inorganics	09	
Organics	12	
Total extractable		21
Insoluble in water (particulate)		
Cellulose	15	
Hemicellulose	15	
Lignin	3	
Unknown	6	

recovered and used as sweetener/simple carbohydrate in crew diet, with no downstream conversion of sugar to single cell protein or myco-protein). This is equivalent to an additional 4 m^2 growing level for the KSC-CELSS breadboard BPC, which is currently configured with 4 such growing levels. These numbers were high enough to justify further exploration of the feasibility of the enzymatic hydrolysis of CELSS crop residue cellulose at the breadboard scale.

In preliminary flask scale studies, Garland and Strayer (unpublished data) have shown that 70% cellulose conversion was possible if the CELSS crop residue had been pretreated with alkaline-peroxide to extract lignin and hemicellulose (2). Although useful for showing the potential for cellulose conversion, this extraction required large quantities of base (NaOH) hydrogen peroxide. With no sure sources for NaOH in an operational CELSS, this pretreatment step was abandoned as untenable. Furthermore, the enzyme was harvested from a 10 day broth culture of *Trichoderma reesei* (QM9414) grown on a purified cellulose source (alpha-cellulose). Brannon and Strayer (unpublished data) eventually adapted this culture to authentic CELSS derived crop residues with cellulase enzyme yields in excess of those using the purer cellulose substrate.

These successful, small-scale flask studies led to larger scale studies of enzyme production in a 10 L fermentor vessel. Preliminary calculations using the results of these studies indicated that multiple batch fermentor runs at the 10 L scale may be sufficient to process the entire inedible biomass output from the KSC breadboard BPC over the same time interval (85 days) required to produce the biomass. This paper reports the results of five replicate runs at approximately half breadboard-scale of both cellulase enzyme production, using *T. reesei* QM9414 grown on CELSS-derived wheat residues, and cellulose hydrolysis that used this enzyme to convert CELSS-derived wheat residue cellulose into glucose.

METHODS: PREPARATION OF INEDIBLE BIOMASS.

After the start of this study, NASA requirements to develop techniques and scale-up mineral recovery (Garland, Mackowiak, and Sager, in preparation) from CELSS materials limited the availability of BPC-grown wheat residues. Also, a number of preliminary breadboard-scale enzyme production runs further depleted this resource. As a result, the runs reported in this paper used leaves and stems of wheat (*Triticum aestivum* cv Yecoro roja) harvested from bench-top greenhouse studies at Utah State University (laboratory of Dr. B. Bugbee). These residues are similar in composition to those grown in the BPC. Residues were dried (1.5 hr at 70°C) and milled to 1-2 mm diameter. Just prior to use as substrate for inoculum preparation, enzyme production, or cellulose hydrolysis, the residues were leached (aqueous extraction, 50 g (dry weight) residue per liter deionized water) for 2 hr at room temperature (3). Higher temperatures were not needed to extract soluble minerals, which is the major function of this procedure at the KSC breadboard facility. The aqueous phase was removed by gravity filtration of the extracted slurry through a stainless steel screen (1.2 mm dia. holes at a density of 56 holes/cm^2) located in the bottom of the extraction vessel. The particulate residues that accumulated on the screen acted as a filter bed. The first liter of filtrate typically contained suspended solids and was refiltered through the retentate. The particulate retentate was rinsed 5 times with 1/5 volume of deionized water.

METHODS: ENZYME PRODUCTION.

INOCULUM PREPARATION - A finding of the preliminary fermentor runs was that reproducible success depended on stringently following an inoculum preparation protocol. The one that was developed followed recommendations in Parton and Willis (4). Briefly, a master, freeze-dried spore suspension was used to periodically inoculate 'first generation' slant cultures. Every two weeks a slant culture was used to inoculate a petri plate working culture.

To initiate an enzyme production run, a 10 ml standardized spore suspension (0.4 Absorbance units at 600 nm) was prepared from the working culture and used to inoculate a 250 ml shake flask containing 100 ml Mandels' medium (5) plus leached wheat residue (25 g [dry weight]/L). The shake flask was incubated at room temperature on a rotary shaker at 120 rpm for 72 hours, sufficient to give an actively growing mycelial culture. This culture was then transferred to fresh medium (10% v/v inoculum) after a brief homogenization to disperse the hyphal clumps. The contents of these flasks were used eventually to inoculate the larger scale fermentor, which required a 10% inoculum of actively growing mycelia. The conditions for the second phase of inoculum growth were the same as the first, i.e., 120 rpm at room temperature for 3 days.

A number of papers have reported that the cellulase complex of *T. reesei* is deficient in beta-glucosidase, the enzyme responsible for converting the disaccharide cellobiose, as well as other oligosaccharides, into glucose (6, 7). Supplementation of the enzyme complex with an outside source of beta-glucosidase is most often used to overcome this deficiency (8). Flask-scale studies of the hydrolysis of CELSS wheat residues confirmed these reports (enzyme produced from alpha cellulose). In addition, it was found that supplemental beta-glucosidase from *Aspergillus phoenicis* QM329, grown on starch, increased glucose yields.

The *A. phoenicis* culture was adapted to CELSS wheat residues as substrate, an inoculum preparation protocol similar to that for *T. reesei* was developed, and the fermentor scale production of beta-glucosidase from CELSS residues was similar to that for *T. reesei*, except that carbon dioxide in the offgas was not monitored.

FERMENTOR VESSEL PREPARATION - Sterizilization (2 hr at 121^{o}C) of the 14 L fermentor vessel (New Brunswick Dual Ferm 320), containing Mandels' medium (5) and leached wheat residue, resulted in flash boil-over of fermentor contents as the autoclave cooled. Unknown quantities of wheat residue substrate were lost and the fermentor entry and exit tubes were clogged with residue. This led to the development of a better method that emloyed separate sterilization of the wheat residue and fermentor plus liquid medium, even though the possibility of contamination was greater. The sterile residue was transferred aseptically to the fermentor under a laminar flow bench, which lessened the chance for airborne contamination.

FERMENTOR ENVIRONMENTAL PARAMETERS - The loaded fermentor, containing 7 to 8 L working volume of Mandels' medium plus 25 g (dry weight) leached wheat residue per L, was inoculated with 10 % v/v of the 3 day old mycelial shake flask cultures. Operational environmental parameters were 0.35 L of sterile air (0.2 um pore membrane filter) per L of liquid fermentor contents, stirring rate of 200 rpm, and room temperature. At the time of this study, pH control and monitoring hardware, including a sterilizable sensor, were not available, so pH was not controlled or monitored. Carbon dioxide concentration in the fermentor offgas was continuously monitored with an infra-red gas analyzer connected to a data collection computer.

ASSAYS - During each fermentor run, samples were collected intermittently and stored (4^{o}C). These samples were subsequently analyzed for cellulase (filter paper assay--glucose end product (5)) and, occasionally, beta-glucosidase activity (5). At 10 to 11 days after inoculation, the fermentor was harvested and samples were taken for dry weight determinations and for assays of cellulase and beta-glucosidase activity. The bulk of the fermentor contents were used to start the next phase/bioreactor--cellulose hydrolysis.

METHODS: ENZYMATIC HYDROLYSIS OF WHEAT RESIDUE CELLULOSE

BIOREACTOR SET-UP - The cellulose hydrolysis bioreactor was a 19 L Wheaton Turbo Lift vessel with a top motor drive system and marine impeller. This system was designed by the manufacturer for mammalian cell suspension culture. Stirring was desired to prevent buildup of potentially inhibitory end products (i.e, glucose) at the active enzyme sites (9, 10) and because the low shear mixing environment should prevent denaturation of the extracellular cellulase enzymes. The vessel was loaded with 500 g (dry weight) leached wheat residues and steam sterilized. After cooling, harvested material from the cellulase (5 L) and beta-glucosidase (2 L) production fermentors were added. Final reactor volume was about 12 liters. A commercial cellulase source (2 runs, Genencor Cytolase 300) was also used for comparison with the CELSS-produced cellulase enzymes.

ENVIRONMENTAL PARAMETERS - The crop residue hydrolysis temperature (50^{o}C), pH (4.8--starting pH adjusted with sterile hydrochloric acid additions), and mixing rate (100 rpm) were obtained from the literature (5), whereas substrate concentration (50 g [dry weight] wheat residue L^{-1}), bioreactor volume (10 - 12 L), and hydrolysis duration (7 days) were selected after small scale preliminary studies. Samples were collected daily for analysis of the desired end product (glucose, glucose oxidase/peroxidase assay, Sigma Chemical). At harvest, the solids were separated and dried overnight (70^{o}C) for determination of dry weight.

RESULTS AND DISCUSSION--ENZYME PRODUCTION

The production of fungal biomass (growth) usually precedes the production of a secondary metabolite (11) such as extracellular cellulase complex. As evidenced by the typical carbon dioxide production curve in FIGURE 2 (for enzyme production run #4), *T. reesei* growth reached maximal rates soon after inoculation with 10% active mycelial culture. Aerobic microbial growth and carbon dioxide production are directly linked, and rates of carbon dioxide production by resting, senescent, or dead cells is very low to zero. The maximal CO_2 production rates in FIGURE 2 persisted for about one day, then gradually declined over the next 3 days. The usual reason for fungal mycelial cultures ending growth and entering a stationary phase are depletion of available substrates and other nutrients and/or accumulation of inhibitory levels of metabolic wastes (12). The limited scope of this study and personnel and logistic limitations precluded a detailed analysis of fermentor broth for the many potential organic compounds that might cause a cessation of fungal growth.

Conversely, cellulase enzyme activity in the culture broth gradually increased from inoculation on day

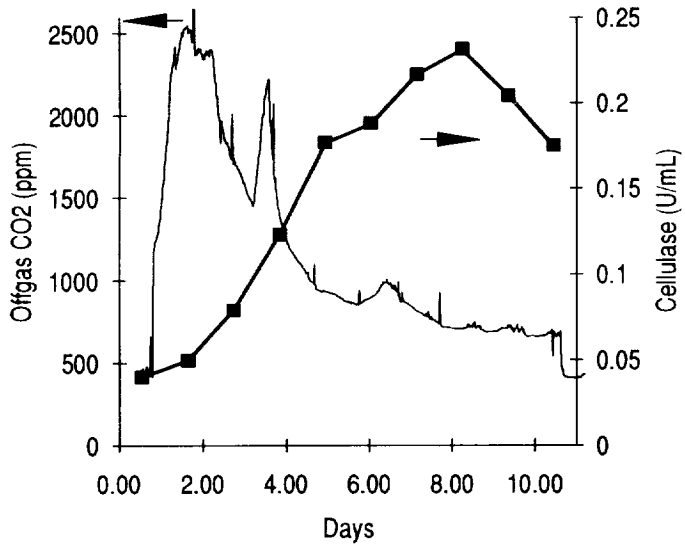

FIGURE 2. TIMECOURSE OF CARBON DIOXIDE PRODUCTION (SOLID LINE--PPM CO_2 CONCENTRATION IN FERMENTOR OFF-GAS) , INDICATING FUNGAL GROWTH, AND CELLULASE PRODUCTION (FILLED SQUARES--CELLULASE ACTIVITY, UNITS/ml) DURING A TYPICAL BREADBOARD-SCALE ENZYME PRODUCTION RUN.

0 to day 7. This indicates that cellulase production was not linked to the growth of the fungus, but was a secondary metabolite. The decline in enzyme activity from day 7 to day 10 could be the result of enzyme inactivation, absorption to particulate substrates, etc. The results of Acebal et al. (13) show nearly identical *T. reesei* growth and cellulase enzyme production over the same time intervals.

A summary of cellulase enzyme production during the five runs is shown on FIGURE 3. Complete data was collected for three of the five runs, with a beginning and end value for run #1 and an intermediate value for run #3. The data show that enzyme production gradually builds from inoculation to day 6 or 7, with three of the five runs showing a decline in enzyme activity by harvest at day 10. The decline in enzyme activity at harvest was as low as 60 % of the maximum at day 7. Considering this decline, most of the runs should have been harvested near day 7 when enzyme levels were greatest.

Because the goal of these studies was to demonstrate feasibility at the breadboard scale, attempts to optimize cellulase enzyme production were not made. Cellulase yields could probably be improved through a thorough study of the effects of environmental parameters. In preliminary fermentor runs, enzyme yields were observed to be negligible when stirring rates were 250 rpm or greater. Shear stress, dissolved oxygen level, or other factors may have been responsible. Another way to increase enzyme yields is to select better strains of cellulolytic microorganisms that have

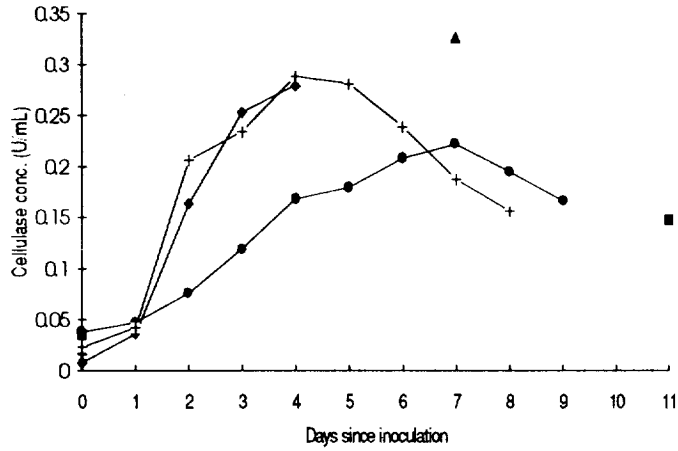

FIGURE 3. TIMECOURSE OF BREADBOARD-SCALE CELLULASE PRODUCTION (ENZYME ACTIVITY, UNITS/mL) FROM CELSS WHEAT RESIDUES BY *TRICHODERMA REESEII* (QM9414) DURING FIVE REPLICATE RUNS. SOLID SQUARES--RUN 1, SOLID DIAMONDS--RUN 2, SOLID TRIANGLES--RUN 3, SOLID CIRCLES--RUN 4, SOLID PLUSES--RUN 5.

been developed to hyperproduce cellulase (14, 15). Strain QM 9414, used in this study, was recommended because it had been the focus of a large amount of research. Last, alternative culture techniques, such as "solid-state" fermentation, may produce higher cellulase yields than the submerged culture method used here (16).

RESULTS AND DISCUSSION--CELLULOSE HYDROLYSIS

The true test of the cellulase enzyme produced from CELSS derived materials is not the short term cellulase activity assay (1 hr with filter paper, a purified cellulose) shown in FIGURES 2 and 3, but its longer term use to hydrolyze the more complex lignocellulose complex found in crops. A summary plot of the five cellulose hydrolysis runs using CELSS produced enzymes and two hydrolysis runs using a commercial cellulase is shown in FIGURE 4. Typically, cellulose hydrolysis was complete by day 3, as evidenced by maximal glucose concentrations. The lowest glucose yield occurred during the run using cellulase from the first CELSS enzyme production run. The next lowest hydrolysis results were obtained using the commercial enzyme. Higher cellulose hydrolysis was obtained with the other four CELSS produced enzyme runs.

The commercial cellulase source (Genencor Cytolase 300) was selected for comparison. This enzyme is marketed primarily for clarification of fruit juices, but the technical representives and product literature state that it can be used for cellulose hydrolysis. The enzyme is produced by a proprietary strain of *T. reesei* (using an undisclosed substrate) and should have been comparable to our enzyme.

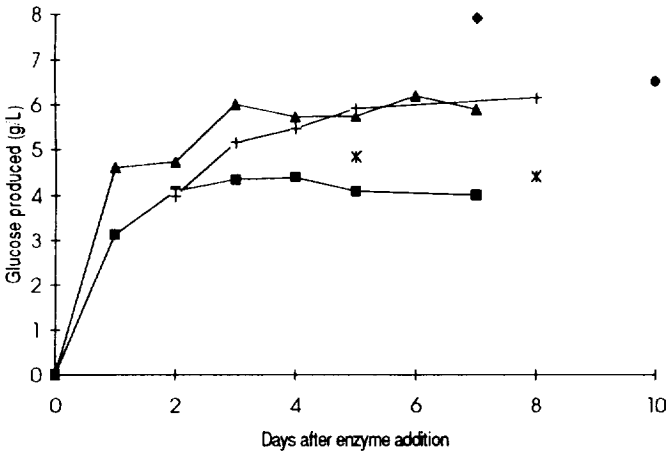

FIGURE 4. TIMECOURSE OF BREADBOARD-SCALE ENZYMATIC HYDROLYSIS OF CELSS WHEAT RESIDUE CELLULOSE (CUMULATIVE GLUCOSE CONCENTRATION, g/L) DURING FIVE REPLICATE RUNS. SOLID SQUARES--RUN 1, SOLID DIAMONDS--RUN 2, SOLID TRIANGLES--RUN 3, SOLID CIRCLES--RUN 4, SOLID PLUSES--RUN 5, ASTERISK--GENENCOR CYTOLASE 300.

The day 3 maximal glucose yield of 6 g L^{-1} resulted from the hydrolysis of cellulose contained in 50 g (dry weight) leached wheat residues L^{-1}. These leached residues had an assumed cellulose content of ca. 38%. Using these values, an overall cellulose conversion efficiency of ca. 29% was calculated. This value was low, but not atypical for residue that still contained lignin and hemicellulose.

Optimization of cellulose hydrolysis at the breadboard scale was not attempted. Improvements in cellulose conversion to glucose can certainly be made, but the associated "costs" of these improvements may not justify their inclusion in a CELSS. As already stated, chemical or physical pretreatment of residue to remove lignin and hemicellulose (3, 17, 18) could at least double the conversion efficiency. The "costs" of these pretreatments might be prohibitive: chemical extractions--hardware (weight, volume) for production of base, peroxide, acid, etc.; and steam explosion--hardware (weight, volume, energy) for producing and containing high pressure steam (1170 to 3240 kPa at 190 to 240°C).

Cellulose conversion efficiency might also be improved by removal of the product glucose. The buildup of glucose has been shown to decrease the activity of the cellulose complex through end-product inhibition (6,7). Tjerneld et al. (19) has shown that continuous, cross-flow ultrafiltration to remove enzymatically produced glucose can increase glucose yields. A potential disadvantage of this process is the lowered concentration of glucose in the filtrate, but this will only be a problem if downstream processing requires a more concentrated substrate.

EVALUATION--SCALE-UP OF CELLULOSE CONVERSION FOR INCLUSION IN THE CELSS BREADBOARD

Many factors could be included in an evaluation of enzymatic cellulose hydrolysis for inclusion in a CELSS. The costs of the processes--increase in weight, volume, energy, and complexity--are disadvantages or negative factors. For this study, enzyme production operating costs per fermentor run were 138 g (dry weight) wheat residue, 5 L working liquid volume, 1.1 L inoculum preparation volume, fungal growth medium constituents, and a relatively low energy consumption (aeration and stirring motor). Operating costs for each cellulose hydrolysis run were 500 g (dry weight) wheat residue, 10 - 12 L working volume, and a moderately high energy consumption (maintenance of 50°C temperature and a stirring motor). The fermentor and bioreactor hardware, including sterilization components, is a fixed cost that should be spread out over the life-time of the hardware. A significant, but hard to evaluate, cost is the increased system complexity. Of noteworthy concern in this area is the requirement to maintain an uncontaminated monoculture of *T. reesei* and the increase in personnel time for process set-up and operation.

The benefit of cellulose conversion to a CELSS is an increase in food per total crop biomass produced m^{-2}. Per cellulose conversion run, a total of 60 - 72 g of glucose was produced from an input of 638 g (dry weight) leached wheat residues, 138 g for enzyme production and 500 g for cellulose hydrolysis. If the batch-mode cellulose conversion processes were run continuously, with minimal downtime between runs, the limiting process would be enzyme production at 7 days. If one day were added to this for fermentor cleanup and sterilization, then 8 days per batch would be possible. From seed to harvest, production of a crop of wheat in the KSC-CELSS breadboard BPC took 85 days (20) , thus ca. 10 cellulose conversion runs (85 days/8days) would be needed to process the entire BPC wheat harvest.

The best BPC wheat yield has been 46 kg total biomass (2.9 kg m^{-2}) of which 28 kg was inedible (e.g., harvest index of 40%). Aqueous extraction (leaching) would remove 35% of this and leave almost 18 kg particulate retentate/filter cake, which would be available for cellulose conversion. Applying the data obtained from this study, the glucose yield from cellulose conversion would range from 1.7 to 2.0 kg and increase the edible yield by 10%, from 18 to 20 kg. This 10 % increase in harvest index is also equivalent to about 6 BPC trays (of 64) or 1.6 m^2 growing area (of 16). The costs for cellulose conversion should thus be compared to the operational and fixed costs required to grow 1.6 m^2 of wheat.

The particulate residues (undigested crop residue and dead and senescent *T. reesei* mycelia) remaining after the cellulose conversion process are a secondary product of these processes and could be

treated further, i.e., combusted or biologically digested (aerobic or anaerobic mixed microbial community or consortia). In preliminary studies by the KSC-CELSS breadboard aquaculture group, these products were incorporated into a tilapia diet, but fish weight gains were minimal (Owens, unpublished data).

CONCLUSION

Breadboard-scale cellulase production from CELSS wheat residues is technically feasible, with sufficient enzyme produced in 7 days to hydrolyze approximately 30 % of the cellulose contained in this residue. This low yield, coupled with the costs involved with generating and using the enzyme, makes this process marginally useful to a CELSS by contributing to a small 10% increase in harvest index. Conversion of the glucose to a more useful product such as single cell protein decreases this benefit even more. Optimization of enzyme production and cellulose hydrolysis, not done for this study, will make these processes more atttractive to CELSS.

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